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The proposal is aimed at utilizing new biosensors based on guanine electron transfer to quantitate messenger RNA for breast cancer genes. The biosensors are based on a scheme involving abstraction of electrons from the guanines of immobilized RNA to generate a signature current for a specific gene. The purpose of the proposed research is to demonstrate that the guanine electron transfer technology can be used to detect overexpressed RNAs in real biological samples. The scope involves detection of the mRNAs for HER-2/neu and FAK, which are both known markers of breast cancer, in samples generated by PCR amplification, reverse transcription, or direct RNA extraction. This report describes experiments that led to detection of the HER-2/neu gene from BT-474 cells using PCR amplification. The research has resulted in a new, more easily automated procedure involving direct adsorption of DNA from a novel combination of solvents. These electrodes allow for the detection of as little as 0.5 fmol of adsorbed DNA per square centimeter of electrode area.

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H. Holmes Hays 7/11/99

PI - Signature Date

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Introduction

The proposal "Applications of a Novel Nucleic Acid Detection Method in Breast Cancer: Analysis of Overexpression of HER-2/neu and FAK" is aimed at utilizing new biosensors based on guanine electron transfer to quantitate messenger RNA for breast cancer genes. The biosensors are based on a scheme involving abstraction of electrons from the guanines of immobilized RNA to generate a signature current for a specific gene. The purpose of the proposed research is to demonstrate that the guanine electron transfer technology can be used to detect overexpressed RNAs in real biological samples. The scope involves detection of the mRNAs for HER-2/neu and FAK, which are both known markers of breast cancer, in samples generated by PCR amplification, reverse transcription, or direct RNA extraction. This report describes experiments that led to completion of the first task in the approved Statement of Work -- detection of the HER-2/neu gene from BT-474 cells using PCR amplification. Changes in the immobilization strategy were required that suggest adjustments in the future tasks that are described in the conclusions.

Body

The sensing technology used in the research is based on electron transfer from guanine in immobilized nucleic acids to a solid electrode.[1] The electron transfer is mediated by a soluble small mediator, $Ru(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine), which greatly enhances the current generated from immobilized nucleic acids by accelerating the electron transfer reaction.[2-5] The mediator is oxidized at the electrode (eq 1) and then abstracts an electron from guanine to regenerate the reduced form and complete a catalytic cycle. In the absence of the mediator, the direct abstraction of electrons from guanine is extremely slow and produces little or no signal.

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$Ru(bpy)_3^{3+} + G \rightarrow Ru(bpy)_3^{2+} + G^+ \quad (2)$$

The sensing strategy is based on immobilizing probes to the electrode where inosine is substituted for guanine, prohibiting electron transfer according to eq 2.[6,7] When the silent inosine probe is hybridized to a large, guanine-containing DNA or RNA target, high concentrations of guanine are generated at the electrode and a large current is generated via the cycle shown in eqs 1 and 2.

Central to using the mediated electron transfer strategy is a means for immobilizing inosine-substituted probes on solid electrodes. We have reported a number of such strategies, and the original proposal called for a promising method we had developed that utilized poly(ethylene terephthalate) (PET) membranes attached to indium tin oxide (ITO) electrodes.[6] We have tested this strategy on biological samples and have found problems with the reproducibility that were not apparent with the model samples. We have therefore developed a new strategy based on direct adsorption for detecting PCR products that is described here and has allowed us to complete Task 1 despite the problems encountered with the PET system. The new direct adsorption strategy has a number of advantages and will be more easily automated than the original PET method.

The immobilization method consists simply of adding purified PCR reactions to 90% dimethyl formamide (DMF) mixtures with 10% acetate buffer and placing over the ITO electrode. After 15 min, the DNA was irreversibly adsorbed to the electrode and could be detected by electron transfer according to eqs 1 and 2.

Results. PCR products were adsorbed to ITO electrodes from 90/10 DMF:acetate buffer with relatively high efficiencies. Double-stranded amplicons of 1497, 1020, and 435 bp were prepared by reverse transcription of the HER-2 mRNA from BT-474 cells (see experimental section below). Results were identical if mRNA from tumor samples was used; however, for developing the method, BT-474 cells were preferable since all sample would show overexpression of HER-2. The PCR fragments were ^{32}P end-labeled and adsorbed onto ITO electrodes. The radioactivity on the electrode was quantitated following extensive rinsing and the extent of adsorption was determined for each fragment at a range of starting DNA concentrations. The percentages of adsorbed DNA are shown in Figure 1 for the three fragments. As shown in the Figure, as much as 35% of the DNA in the starting solution of the 1497 bp fragment remained attached to the electrode following rinsing.

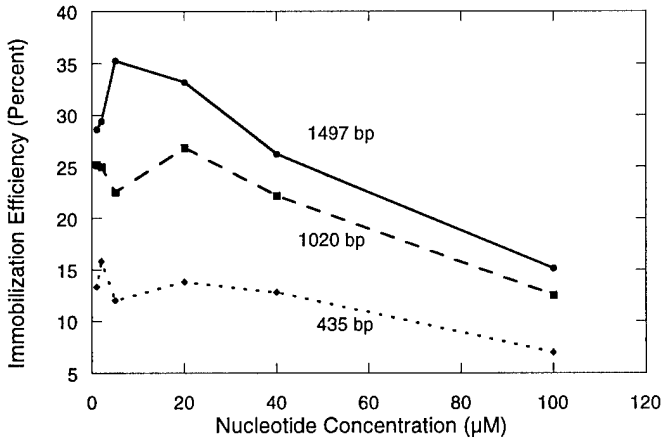

Figure 1. Percentage of DNA applied to electrodes adsorbed from 90/10 DMF/acetate for fragments of 1497 (circles), 1020 (squares), and 435 (diamonds) base pairs. The DNA was prepared by RT-PCR of the HER-2 mRNA from BT-474 cells. The fragments were radiolabeled and the quantity adsorbed was determined using a phosphorimager.

The immobilized electrodes were then exposed to a solution of $Ru(bpy)_3^{2+}$, and the solutions were analyzed by cyclic voltammetry and chronoamperometry. In the case of cyclic voltammetry (Figure 2), a low current was obtained for $Ru(bpy)_3^{2+}$ alone, and larger currents were obtained for electrodes treated with more concentrated solutions of DNA. The peak currents were determined for electrodes modified with varying quantities of the three fragments. As expected, fewer strands of the longer fragments produced larger catalytic currents, because the longer DNAs contained more guanines

and therefore gave more catalysis. Thus, there was a different dependence of the peak current on the quantity of strand immobilized for each fragment (Figure 3), but the slope became steeper as the fragment became longer.

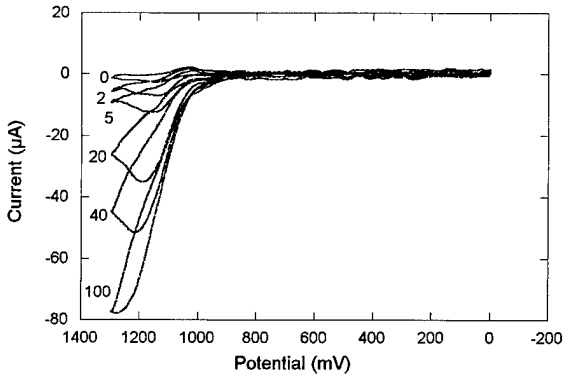

Figure 2. Cyclic voltammograms of $Ru(bpy)_3^{2+}$ at electrodes modified with increasing amounts of DNA. Electrodes were treated with solutions of the 1497 bp fragment that were 0, 2, 5, 20, 40, and 100 μM in nucleotide.

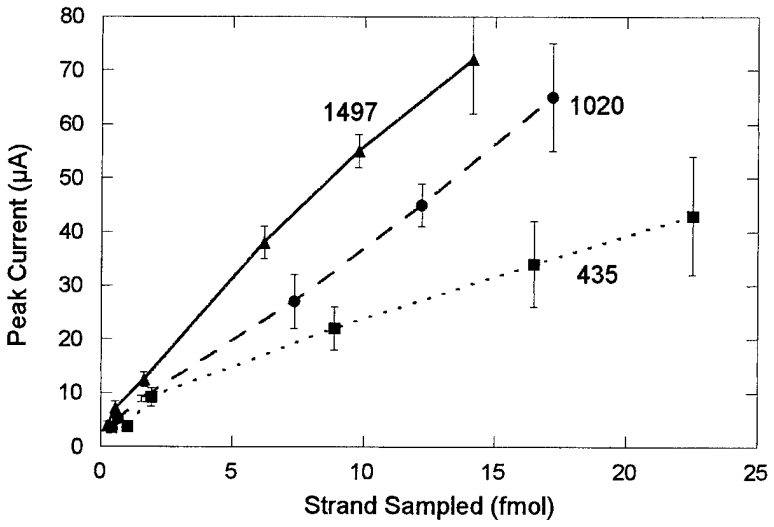

Figure 3. Dependence of the peak current from cyclic voltammograms of $Ru(bpy)_3^{2+}$ at electrodes modified with varying quantities of the 1497, 1020, and 435 bp fragments. Error bars represent standard deviations from multiple trials. The quantities of adsorbed DNA were determined by radiolabeling followed by analysis with a phosphorimager.

The results in Figure 3 show that for a given fragment, a quantitative relationship is obtained between the peak current and the amount of strand immobilized on the electrode. We have therefore demonstrated, according to approved Task 1, that we can detect RT-PCR product of HER-2 by the electrochemical method. The sensitivity of the method shown in Figure 3 (defined as three times the standard deviation of the signal from $Ru(bpy)_3^{2+}$ obtained in the absence of DNA) is 0.5 fmol of immobilized strand per 0.1 cm^2 of ITO electrode. This level is approaching that of radiolabeling and phosphorimagery with much greater simplicity. The reproducibility and sensitivity of the method are superior to that originally attempted with the PET membranes, and we therefore feel strongly that we should move forward using this new procedure. Recommended changes in the statement of work are discussed in the Conclusions section, although we feel that with this new method, completion of the primary tasks of detecting messenger RNA from HER-2 in cancer cells remains feasible during the grant period and that the data in Figure 3 constitute considerable progress toward these goals.

Experimental. BT-474 cells were provided by our collaborators in the laboratory of Dr. Bill Cance at the UNC Lineberger Comprehensive Cancer Center. RNA was extracted and purified by means of an RNeasy RNA purification kit from Qiagen. Reverse transcription was performed by mixing 2 μL of total RNA (0.8 μg/μL), 2 μL random hexamers (Promega), 4 μL dNTP's (10 mM, Amersham) and 6 μL RNase-free water. The reverse transcription mix was heated to 60°C for 3 min to denature the RNA and then immediately placed on ice. To the mix was added 1 μL RNasin (Promega), 1 μL M-MLV Reverse Transcriptase (Life Technologies, 200 U/μL), and 4 μL 5X Reverse Transcription Buffer (Life Technologies). The reactants were mixed and incubated at 37°C for 1 hr.

A 50 μL PCR mix was assembled from 5 μL of the reverse transcription reaction, 2.5 μL of the forward primer (5 μM), 2.5 μL of the reverse primer (5 μM), 2.5 μL $MgCl_2$ (50 mM), 2.5 μL dNTP's (10 mM), 5 μL 10X PCR buffer, 30 μL water, and 1 U Taq polymerase (Life Technologies, 5 U/μL). The 435 bp product (forward primer, GGC TGT GCC CGC TGC AAG GGG CCA; reverse primer, GCA GCC AGC AAA CTC CTG GAT ATT) was produced as follows: 1 cycle at 95°C for 5 min, 30 cycles at 94°C for 20 sec, 55°C for 30 sec, 72°C for 40 sec, and 1 cycle at 72°C for 5 min. The 1,020 bp product (forward primer, GGC TGT GCC CGC TGC AAG GGG CCA; reverse primer, CGG CAA ACAGTG CCT GGC ATT) was produced as follows: 1 cycle at 95°C for 5 min, 30 cycles at 94°C for 20 sec, 64°C for 30 sec, 72°C for 1.5 min, and 1 cycle at 72°C for 10 min. The 1,497 bp PCR product (forward primer, GGC TGT GCC CGC TGC AAG GGG CCA; reverse primer, CCT CAG CTCCGT CTCTTT CAG) was synthesized as follows: 1 cycle at 95°C for 5 min, 30 cycles at 94°C for 20 sec, 62°C for 30 sec, 72°C for 2 min, and one cycle at 72°C for 10 min. Each PCR product was tested for purity by gel electrophoresis on a 2% agarose gel. Both the 435 and 1,497 bp sequences yielded one product of the appropriate size as determined on the gel. The 1,020 bp sequence constantly yielded a low molecular weight band (< 200 bp) that was less than 1% as intense as the major band of the appropriate size observed on the gel. Primers and unincorporated nucleotides were removed from the RT-PCR products via a Concert PCR

Purification kit (Life Technologies). The purified products were used as templates for further amplifications as more product was needed.

The purified PCR products that were to be used for electrochemical detection were ethanol precipitated by adding 5 M ammonium acetate to the purified products to reach a final concentration of 0.5 M ammonium acetate and then adding three volumes of ice cold ethanol. The PCR products were then placed on dry ice for 20 - 30 min. Following precipitation, the PCR products were pelleted by centrifugation at maximum speed for 20 min in a refrigerated microcentrifuge (Fischer). The ethanol supernatant was carefully removed, and the pellet was dried on a speed vac (Savant). The pellets were resuspended in 100 mM sodium acetate buffer at pH = 6.8. This procedure typically yielded about 70 μL of 1 mM PCR product (concentration determined in moles of nucleotide).

Tin doped indium oxide coated electrodes were cleaned according to the following procedure. ITO electrodes were sonicated in an alconox solution (8 g alconox per liter water) for 15 min. The electrodes were rinsed and sonicated in isopropanol for 15 min followed by two 15 min sonications in water. The electrodes were then allowed to air dry. Purified PCR products were immobilized onto the ITO surface by mixing 5 μL of the PCR product in 100 mM sodium acetate buffer, pH = 6.8, with 45 μL dimethyl formamide (DMF). The 50-μL mixture was pipetted onto the ITO, completely covering the electrode. The electrode was placed in a constant humidity chamber for one hour because preliminary experiments with the system showed that a maximum amount of calf thymus DNA was immobilized on the ITO by this method within 15 minutes. The electrodes were then rinsed by immersing them in solutions that were agitated on a rotary mixer (Thermolyne). The electrodes under went two water washes each one for 3 min, one wash in 1 M sodium chloride for 5 min, one wash in 100 mM sodium phosphate (pH = 7.0) for 5 min, and three final washes in water each for 3 min. The electrodes were then allowed to dry. The amount of DNA attached to the ITO surface was controlled by changing the concentration of the PCR product that was applied to the ITO.

Electrochemical detection of the surface immobilized PCR products was performed on a BAS 100 B/W potentiostat connected to a 200 MHz pentium computer. All experiments were performed in a one-compartment electrochemical cell. The electrode's geometric surface area was calculated to be 12.6 mm^2. A mini Ag/AgCl (Cypress Systems) electrode was used as the reference, and a platinum wire was used as the counter electrode. Cyclic voltammetry was performed from 0 to 1,300 mV vs Ag/AgCl at a scan rate of 10 V/s. For each batch of electrodes cleaned, one electrode was used to perform a background scan. This electrode was not treated with DNA; however, cyclic voltammetry was performed on it under the above conditions in 50 mM sodium phosphate, pH = 7.0. Cyclic voltammetry was performed on the DNA-modified electrodes according to the parameters above in a solution of 10 μM $Ru(bpy)_3^{2+}$ in 50 mM sodium phosphate, pH = 7.0. The cyclic voltammograms of buffer only were subtracted from the cyclic voltammograms of the DNA-modified electrodes, and the peak currents were measured. Cyclic voltammograms that produced negative signals upon subtraction were not used in further analysis. Poor background subtraction only became a significant source of error at final DNA concentrations of 5 μM (concentration of nucleotides in the buffer/DMF mixture) and below.

Radiolabelled PCR products were synthesized according to the method of Mertz and Rashtchian.[8] Briefly, a 20-μL PCR mixture was prepared containing: 1 μL template DNA (100 pg/μL), 1 μL $MgCl_2$ (10 mM), 1 μL dNTP's (100 μM), 2 μL 10 X PCR buffer, 3 μL α-P^{32} dCTP (1.8 μM), and 12 μL water. PCR for each product was performed according to the reaction conditions described above. To determine if the radiolabelled product was synthesized a parallel reaction was performed for each sized fragment of DNA without the α-P^{32} dCTP added. These parallel reaction products were run on a 5% native polyacrylamide gel, stained with sybr-green and visualized under UV illumination beside a 100 bp DNA ladder. All of the parallel reactions yielded one visible product that was the appropriate length. The radiolabelled PCR products were purified on Concert PCR purification cartridges, ethanol precipitated, and dried on a speed vac. To each dried radiolabelled PCR product was added 40 μL of 1 mM PCR product of the same length in 100 mM sodium acetate buffer, pH = 6.8. The newly doped PCR products were used within 1 day of synthesis to minimize the effects of radiolysis.

The doped PCR products were attached to the ITO surface in an identical manner to that of the non-labeled samples and were washed identically also. After the electrodes dried, they were wrapped in plastic wrap and placed on a phosphorimager screen (Molecular Dynamics). One-μL aliquots of certain concentrations from the labeled PCR products were used as quantification standards. These aliquots were applied to filter paper, dried, wrapped in plastic wrap, and placed on the phosphorimager screen. The phosphorimager screen was exposed for approximately 12 hours and scanned on a Molecular Dynamics Storm 840 phosphorimager.

Research Accomplishments

- New method for adsorption of double-stranded DNA to ITO electrodes. The method involves no coupling agents or reactive additives, and can be performed rapidly with no special handling.
- Detection of adsorbed DNA without labeling using catalytic electrochemistry according to eq 2. The sensitivity is 0.5 fmol of strand per 0.1 cm^2.
- Detection of HER-2 mRNA following RT-PCR amplification from breast cancer cells.

Reportable Outcomes

- Results from this report will be submitted as a paper to Analytical Chemistry during the next granting period and should be in print prior to the next annual report.
- The Principal Investigator will report on these and the findings from the next period at the DoD meeting in Atlanta in June, 2000.

Conclusions

The collected data in Figures 1-3 demonstrate a new, robust method for detecting double-stranded DNA produced by PCR. This method applies well to RT-PCR amplification of the HER-2 mRNA from BT-474 cells, a cancer cell line in which HER-2 overexpression is well established. These accomplishments constitute the completion of

approved Task 1. The second task in the approved statement of work is to perform similar studies using strand displacement amplification (SDA). However, some time was lost in replacing the PET membrane method with the present DMF/acetate method. Further, the goals of the research are to perform the analyses without amplification, so we recommend deleting Task 2 from the statement of work. The present reproducibility and sensitivity of the method should be sufficient to proceed on to the detection of cDNA, as described in Task 3. We suggest that by moving on to Task 3, the project can remain on schedule with no loss of progress towards the ultimate goal of detecting unamplified mRNA in clinical samples.

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Appendices

None attached